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Genetic Divergences and Phylogenetic Relationships Among the *Fejervarya limnocharis* Complex in Thailand and Neighboring Countries Revealed by Mitochondrial and Nuclear Genes

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To clarify the genetic divergence in the *F. limnocharis* complex from Thailand and neighboring countries and to elucidate the phylogenetic problems of this taxon, we analyzed partial sequences of the mitochondrial 12S and 16S rRNA genes and the nuclear CXCR4, NCX1, RAG-1, and tyrosinase genes. The *F. limnocharis* complex from Thailand had three distinct haplotypes for 12S and 16S rRNA genes. Nucleotide similarities and the phylogenetic relationships indicated that the haplotype 1 group corresponded to the real "*F. limnocharis*", the haplotype 2 group was *F. orissaensis* or closely related to it, and the haplotype 3 group was possibly an undescribed species. Mitochondrial gene data also showed two major clades of the genus *Fejervarya*, the Southeastern and South Asian groups. Although *F. orissaensis* is so far known only from Orissa in India, the haplotype 2 group was observed in Thailand. This distribution pattern and the phylogeny suggested that the origin of *F. orissaensis* and the haplotype 2 group might lie in Southeast Asia. There was also evidence suggesting that the haplotype 3 group originated in the South Asian area and has spread to northern Thailand. The nuclear gene data did not support the monophyly of the haplotypes recognized by mitochondrial genes. This incongruence between the mitochondrial and nuclear data seems to be caused by ancestral polymorphic sites contained in nuclear genes. Although neither the mitochondrial nor the nuclear data clarified intergeneric relationships, the nuclear data rejected the monophyly of the genus *Fejervarya*.

Key words: sequence divergence, molecular phylogeny, mitochondrial genes, nuclear genes, *Fejervarya*, Thailand

INTRODUCTION

Among anuran species, *Fejervarya limnocharis* is one of the most widely distributed species in Asia, extending from Japan in the east to Nepal in the west and Indonesia to the south (Frost, 1985). Because of few morphological differences, "*F. limnocharis*" has been conventionally regarded as a single species. However, recent detailed analyses have demonstrated that there is a degree of genetic differentiation within conventional *F. limnocharis*, and therefore it has been suggested that "*F. limnocharis*" contains several cryptic species (Dubois and Ohler, 2000). For example, Dubois (1975) concluded that Nepalese "*F. limnocharis*" could be classified into four distinct species. Veith et al. (2001) also described a cryptic species in the *F. limnocharis* complex from Java, Indonesia, and named it *F. iskandari*. Consequently, there are now regarded to be 32 species for the genus *Fejervarya* (Frost, 2006). Thus, the *F. limnocharis* group to be identified should be called the *Fejervarya limnocharis* complex (Djong et al., 2007). Furthermore, there are few morphological differences and few morphological characteristics usable for classification throughout this genus, not only for the *F. limnocharis* complex, and so it is difficult to correctly identify

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species. Therefore, in some cases, even a systematically and greatly different lineage might be included in the *F. limnocharis* complex.

Recently, Kurabayashi et al. (2005) suggested that the genus *Fejervarya* is divided into two main groups, the *F. limnocharis* group distributed in Southeast and East Asia and the *F. syhadrensis* group distributed in India and South Asia. According to Frost et al. (2006), on the other hand, the members of the South Asian *Fejervarya* group form a monophyletic group not with Southeast Asian *Fejervarya* species but with the members of other genera such as *Hoplobatrachus* and *Sphaerotheca*.

Genetic analyses using allozymes and mitochondrial DNA have been carried out for several populations of the *F. limnocharis* complex in Thailand (Sumida et al., 2007; Djong et al., 2007). Both allozyme and mtDNA analyses revealed that the Bangkok population differed greatly from those of *F. limnocharis* from the type locality, Java, Indonesia. In addition, in the allozyme analysis, the Ranong population was more closely related to the Bangkok population than to the Java population in the type locality, whereas in the mtDNA analysis, the Ranong population was more closely related to the Java population in the type locality than to the Bangkok population. Therefore, a possible mtDNA introgression was suggested for the Ranong population (Sumida et al., 2007). At present, however, with regard to the *F. limnocharis* complex in Thailand, the following three questions have not been investigated: (1) how many cryptic species exist, (2) what phylogenetic relationships exist between species of the complex and other *Fejervarya* species, and (3) does hybridization occur among cryptic species (including mtDNA introgression)?

To elucidate these problems, we analyzed two mt genes (the 12S and 16S rRNA genes) and four nuclear genes (CXCR4, NCX1, RAG-1, and tyrosinase). We examined the sequence data for genetic differentiation of the *F. limnocharis* complex in Thailand and neighboring countries, and also examined the phylogenetic relationships among three genera (*Fejervarya*, *Hoplobatrachus*, and *Sphaerotheca*) that were considered possibly nested within the paraphyletic genus "*Fejervarya*" (Frost et al., 2006).

MATERIALS AND METHODS

Specimens

The present study included 86 individuals of the *F. limnocharis* complex from 27 localities in Thailand, Malaysia, Laos, Indonesia, and India (Fig. 1, Table 1). Specimens were stored at the Graduate School of Human and Environmental Studies, Kyoto University

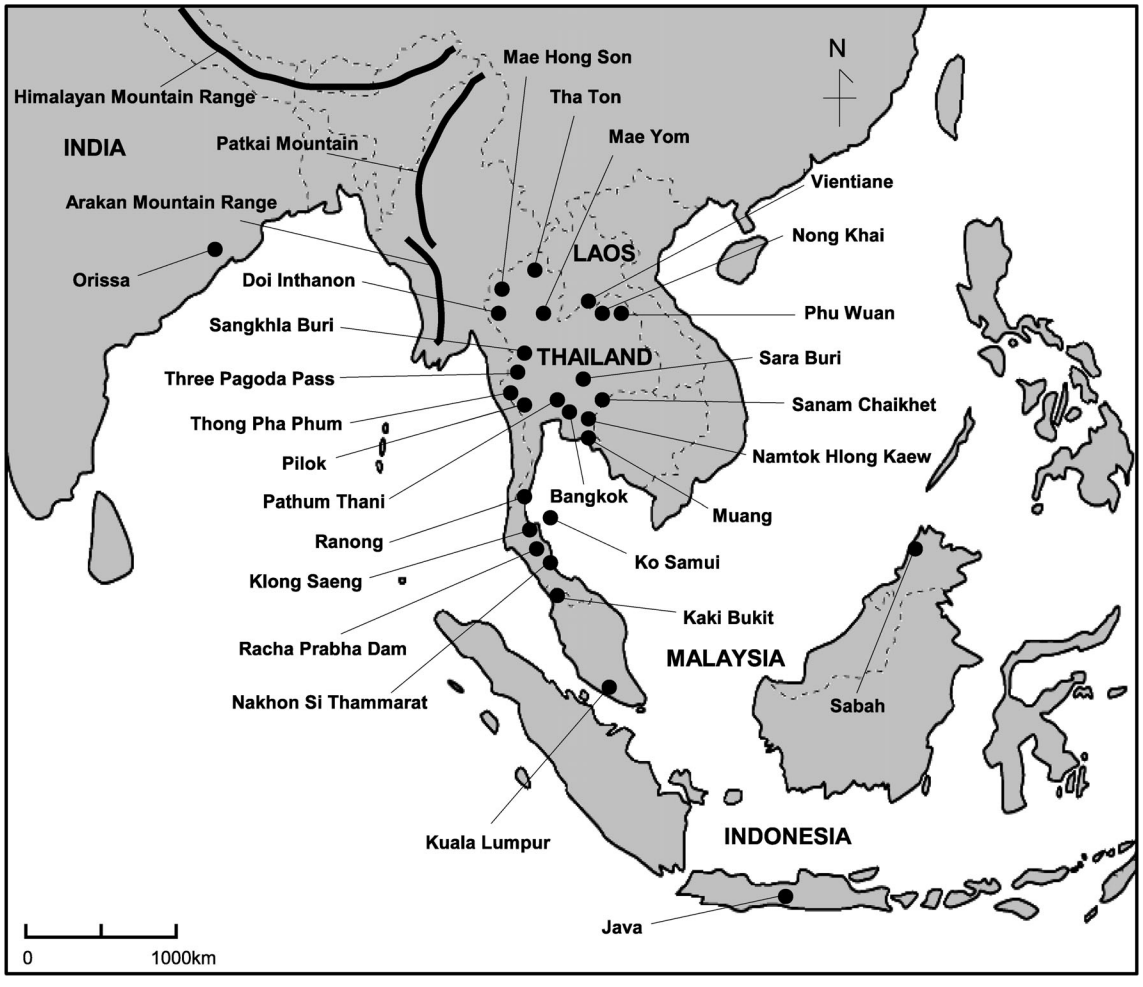

Fig. 1. Map showing the collecting stations for frogs used in the present study.

Table 1. Specimens used and haplotypes observed among nucleotide sequences of the mitochondrial 12S and 16S rRNA genes.

Species	Collecting station		No. of Frogs	Haplotypes(No. of frogs)			
	Country	Locality		12S rRNA	Accession No.	16S rRNA	Accession No.
F. limnocharis	Thailand	Tha Ton	3	12S-Thai 1-1 (3)	AB277275	16S-Thai 1-1 (2)	AB277292
						16S-Thai 1-2 (1)	AB277293
F. limnocharis	Thailand	Mae Hong Son	8	12S-Thai 1-1 (5)	AB277275	16S-Thai 1-1 (5)	AB277292
				12S-Thai 1-2 (2)	AB277276	16S-Thai 1-2 (1)	AB277293
				12S-Thai 2-2 (1)	AB277282	16S-Thai 1-3 (1)	AB277294
						16S-Thai 2 (1)	AB277299
F. limnocharis	Thailand	Doi Inthanon	6	12S-Thai 1-1 (5)	AB277275	16S-Thai 1-1 (5)	AB277292
				12S-Thai 1-3 (1)	AB277277	16S-Thai 1-4 (1)	AB277295
F. limnocharis	Thailand	Mae Yom	2	12S-Thai 1-1 (2)	AB277275	16S-Thai 1-1 (2)	AB277292
F. limnocharis	Thailand	Three Pagoda Pass	2	12S-Thai 2-2 (2)	AB277282	16S-Thai 2 (2)	AB277299
F. limnocharis	Thailand	Pilok	5	12S-Thai 2-1 (2)	AB277281	16S-Thai 2 (2)	AB277299
				12S-Thai 3 (3)	AB277284	16S-Thai 3 (3)	AB277300
F. limnocharis	Thailand	Ko Samui	2	12S-Thai 1-4 (2)	AB277278	16S-Thai 1-1 (2)	AB277292
F. limnocharis	Thailand	Klong Saeng	4	12S-Thai 1-4 (4)	AB277278	16S-Thai 1-1 (4)	AB277292
F. limnocharis	Thailand	Racha Prabha Dam	1	12S-Thai 1-4 (1)	AB277278	16S-Thai 1-1 (1)	AB277292
F. limnocharis	Thailand	Nakhon Si Thammarat	2	12S-Thai 1-1 (2)	AB277275	16S-Thai 1-1 (1)	AB277292
						16S-Thai 1-5 (1)	AB277296
F. limnocharis	Thailand	Sanam Chaikhet	2	12S-Thai 1-1 (2)	AB277275	16S-Thai 1-6 (1)	AB277297
						16S-Thai 1-7 (1)	AB277298
F. limnocharis	Thailand	Nong Khai	2	12S-Thai 1-1 (2)	AB277275	16S-Thai 1-1 (1)	AB277292
						16S-Thai 1-6 (1)	AB277297
F. limnocharis	Thailand	Phu Wuan	2	12S-Thai 1-1 (2)	AB277275	16S-Thai 1-1 (2)	AB277292
F. limnocharis	Thailand	Muang	1	12S-Thai 1-5 (1)	AB277279	16S-Thai 1-1 (1)	AB277292
F. limnocharis	Thailand	Namtok Hlong Kaew	1	12S-Thai 1-5 (1)	AB277279	16S-Thai 1-1 (1)	AB277292
F. limnocharis	Thailand	Sangkhla Buri	2	12S-Thai 1-1 (1)	AB277275	16S-Thai 1-6 (1)	AB277297
				12S-Thai 2-2 (1)	AB277282	16S-Thai 2 (1)	AB277299
F. limnocharis	Thailand	Bangkok	5	12S-Thai 2-1 (5)	AB277281	16S-Thai 2 (5)	AB277299
F. limnocharis	Thailand	Ranong	2	12S-Thai 1-4 (2)	AB277278	16S-Thai 1-1 (2)	AB277292
F. limnocharis	Thailand	Thong Pha Phum	2	12S-Thai 2-1 (2)	AB277281	16S-Thai 2 (2)	AB277299
F. limnocharis	Thailand	Sara Buri	3	12S-Thai 2-1 (2)	AB277281	16S-Thai 2 (3)	AB277299
				12S-Thai 2-3 (1)	AB277283		
F. limnocharis	Thailand	Pathum Thani	12	12S-Thai 2-1 (12)	AB277281	16S-Thai 2 (12)	AB277299
F. limnocharis	Malaysia	Kaki Bukit	2	12S-Thai 1-6 (2)	AB277280	16S-Thai 1-1 (2)	AB277292
F. limnocharis	Malaysia	Sabah	2	12S-Thai 1-1 (1)	AB277275	16S-Thai 1-1 (2)	AB277292
				12S-Thai 1-6 (1)	AB277280		
F. limnocharis	Malaysia	Kuala Lumpur	4	12S-Thai 1-1 (4)	AB277275	16S-Thai 1-1 (3)	AB277292
						16S-Malay (1)	AB277301
F. limnocharis	Laos	Vientiane	1	12S-Thai 1-1 (1)	AB277275	16S-Thai 1-1 (1)	AB277292
F. limnocharis	Indonesia	Java	2	12S-limno1-1 (1)	AB277285	16S-Thai 1-1 (1)	AB277292
				12S-limno 1-2 (1)	AB277286	16S-limno (1)	AB277302
F. iskandari	Indonesia	Java	2	12S-isk (2)	AB277287	16S-isk (2)	AB277303
F. orissaensis	India	Orissa	2	12S-ori 1 (1)	AB277288	16S-ori (2)	AB277304
				12S-ori 2 (1)	AB277289		
S. dobsoni	India	Bajipe	1	12S-dob (1)	AB277290	16S-dob (1)	AB277305
L. laticeps	Malaysia	Kuala Lumpur	1	12S-lati (1)	AB277291	16S-lati (1)	AB277306
Total			86				
F. cancrivora	Philippines				AB070730 [a]		AB070738 [a]
F. vittigera	Philippines				AY313683 [b]		AY313683 [b]
F. syhadrensis	Sri Lanka				AY141843 [c]		AB167948 [c]
F. nilagirica	India	Kudremukh			AB167921 [c]		AB167950 [c]
F. brevipalmata	India	Madikeri			AB167918 [c]		AB167946 [c]
F. rufescens	India	Mangalore			AB167917 [c]		AB167945 [c]
F. sp. hpB	India	Madikeri			AB167924 [c]		AB167954 [c]
H. tigerinus	India	Mangalore			AB167916 [c]		AB167944 [c]

[a]Sumida et al. (2002); [b]Evans et al. (2003); [c]Kurabayashi et al. (2005).

(KUHE) or Institute for Amphibian Biology, Hiroshima University (IABHU) (Appendix 1). Specimens from type localities in Indonesia and India were clearly identified as *F. limnocharis* and *F. iskandari* from Indonesia, and as *F. orissaensis* from India. We also used three species belonging to closely related genera: *Hoplobatrachus tigerinus*, *Sphaerotheca dobsoni*, and *Limnonectes laticeps*.

PCR and sequencing

Total genomic DNA was extracted from muscle tissues using a DNA extraction kit (DNeasy Tissue Kit, Qiagen) according to the manufacturer's protocol. Partial fragments of the mitochondrial 12S and 16S rRNA genes and four nuclear genes, chemokine receptor 4 (CXCR4), Na^+/Ca^{2+} exchanger (NCX1), recombination activating gene (RAG-1), and tyrosinase, were PCR-amplified from the total DNA. Primers used in this study are listed in Table 2. PCR mixtures were prepared with an Ex-Taq Kit (TaKaRa) according to the manufacturer's protocol. Portions of the 12S and 16S rRNA genes from 86 individuals were directly sequenced by using an automated sequencer (3100-Avant, ABI). Three distinct haplotypes were found for the mt genes from individuals of conventional *F. limnocharis*. We then sequenced portions of four nuclear genes from 16 individuals of the *F. limnocharis* complex as representatives of these distinct haplotype groups found in the mt genes sequences (Table 3 and Results). Furthermore, partial nucleotide sequences of the four nuclear genes were determined for *H. tigerinus*, *S. dobsoni*, and *L. laticeps*. Nucleotide sequences obtained in this study were deposited in the DNA Data Bank of Japan (DDBJ) nucleotide sequence database under Accession Nos. AB277275–AB277359 (Tables 1 and 3).

Table 2. Primers used in the present study for PCR amplification.

Gene	Primer name	Sequence (5'-3')	Source
12S rRNA	FS01	AACGCTAAGATGAACCCTAAAAAGTTCT	Sumida et al. (2002)
	R16M1	GGGTATCTAATCCCAGTTTG	Sumida et al. (2002)
16S rRNA	F51	CCCGCCTGTTTACCAAAAACAT	Sumida et al. (2002)
	R51	GGTCTGAACTCAGATCACGTA	Sumida et al. (2002)
CXCR4	CXCR4-Fow1	GTNATGGGCTAYCARAARAA	This study
	CXCR4-Fow2	ATGACWACAAATACAGRYTGCAYCTNTC	This study
	CXCR4-Rev1	TTGAAYTTGGCNCCSAGGAARGCRTA	This study
	CXCR4-Rev2	TAATAAGGMARCCARCAGGYRAARAA	This study
NCX1	NCX1-Fow1	GARAAGGARATAACNATYAARAARCC	This study
	NCX1-Fow2	ATTGAAGTKTGTGGCCAYAAYTT	This study
	NCX1-Rev1	TTTTCATCTTCYTCAAADATRTCRTC	This study
	NCX1-Rev2	TCCTTCTGKGTCTCACCWGGYTTRAA	This study
RAG1	RAG1_Ex1_Fow1	AAATWCTCRGAMTGGAAGTTYAARCT	This study
	RAG1_Ex1_Rev1	TCACCWYCTTCTTCYTTBTCDGCRAA	This study
Tyrosinase	Tyr 1A	AGGTCCTCTTRAGCAAGGAATG	Bossuyt and Milinkovitch (2000)
	Tyr 1E	GAGAAGAAAGAWGCTGGGCTGAG	Bossuyt and Milinkovitch (2000)

Table 3. Accession numbers for nucleotide sequences of the four nuclear genes included in this study.

Species	Collecting station		No. of frogs	Accession Nos.			
	Country	Locality		CXCR4	NCX1	RAG1	Tyrosinase
F. limnocharis	Thailand	Tha Ton	1	AB277307	AB277322	AB277334	AB277348
F. limnocharis	Thailand	Mae Hong Son	1	AB277397	AB277321	AB277335	AB277349
F. limnocharis	Thailand	Three Pagoda Pass	1	AB277308	AB277323	AB277335	AB277349
F. limnocharis	Thailand	Nakhon Si Thammarat	1	Ab277307	AB277321	AB277336	AB277347
F. limnocharis	Thailand	Sanam Chaikhet	1	AB277309	AB277321	AB277337	AB277347
F. limnocharis	Thailand	Bangkok	1	AB277307	AB277323	AB277338	AB277350
F. limnocharis	Thailand	Ranong	2	AB277307	AB277321	AB277333	AB277351
				AB277305	AB277324	AB277333	AB277351
F. limnocharis	Thailand	Sara Buri	1	AB277311	AB277321	AB277339	AB277347
F. limnocharis	Thailand	Pilok	3	AB277312	AB277325	AB277340	AB277352
				AB277313	AB277326	AB277340	AB277353
				AB277314	AB277326	AB277340	AB277353
F. limnocharis	Indonesia	Java	1	AB277315	AB277327	AB277341	AB277354
F. iskandari	Indonesia	Java	1	AB277316	AB277328	AB277342	AB277355
F. orissaensis	India	Orissa	2	AB277317	AB277329	AB277343	AB277356
				AB277317	AB277329	AB277343	AB277356
H. tigerinus	India	Mangalore	1	AB277319	AB277331	AB277345	AB277358
S. dobsonii	India	Bajipe	1	AB277318	AB277330	AB277344	AB277357
L. laticeps	Malaysia	Kuala Lumpur	1	AB277320	AB277332	AB277346	AB277359
Total			19				

Phylogenetic analyses

The nucleotide sequences of each gene from 16 *Fejervarya* individuals (five individuals of haplotype 1 group, four individuals of haplotype 2 group, three individuals of haplotype 3 group, *F. limnocharis*, *F. iskandari*, and two individuals from *F. orissaensis*) and those of three other species (*H. tigerinus*, *S. dobsoni*, and *L. laticeps*) were aligned using the program ClustalW (Thompson et al., 1994). For mt gene sequences, we also added the sequence data for 10 *Fejervarya* species whose data were usable from the database. To exclude gaps and ambiguous sites, we revised the alignments using GBlock 0.91b (Castresana, 2000) with the default settings. We combined the two mt rRNA gene sequences (total of 638 sites) and made a concatenated alignment of four nuclear genes (total of 2650 sites). Based on two concatenated alignments of the mitochondrial and nuclear genes, phylogenetic analyses were performed by the maximum-likelihood (ML) and maximum-parsimony (MP) methods implemented in PAUP* 4.10b (Swofford, 2002). We also carried out Bayesian inference (BI) by using MrBayes ver. 3.0b4 (Huelsenbeck and Ronquist, 2001). The partition homology test (Farris et al. 1995) did not reject concordant phylogenetic signals between two the mt rRNA genes but rejected the concordance among the four nuclear genes. Thus, in BI analyses, we treated the four nuclear genes as different partitions. For the BI analyses, the following settings were applied: number of Markov chain Monte Carlo (MCMC) generations=two million and sampling frequency=10, with the first 200,000 generations discarded. For ML and BI analyses, best-fit substitution models were chosen by the Akaike information criterion implemented in MODELTEST ver. 3.06 (Posada and Crandall, 1998), as follows: GTR+I+G for the concatenated mt gene data (ML and BI analyses); GTR+I+G for the concatenated nuclear gene data (ML); TrN for the CXCR4 and NCX1 partitions, and GTR for the RAG1 and tyrosinase partitions (BI). The reliabilities of the resultant phylogenetic trees were evaluated with the bootstrap proportion (BP). BP values were calculated by analysis of 100 pseudoreplicates for the ML analysis and of 1000 pseudoreplicates for the MP analysis. Statistical support for the resultant BI trees was determined by Bayesian posterior probabilities (BPP). The topologies of the resultant trees and several alternative ones were compared by resampling of the sitewise log-likelihoods (RELL), i.e., the Kishino-Hasegawa (KH: Kishino and Hasegawa, 1989) and Shimodaira-Hasegawa (SH: Shimodaira and Hasegawa, 1999) tests, implemented in PAUP*. RELL was conducted with 10,000 resamplings.

RESULTS

Mitochondrial 12S and 16S rRNA genes

Nucleotide sequences were determined for partial portions of the 12S and 16S rRNA genes from 86 individuals including the *F. limnocharis* complex from Thailand and neighboring countries. The surveyed *F. limnocharis* complex from Thailand had three haplotypes for the mt genes (Figs. 2 and 3). Haplotype 1 was found from a wide region in Thailand, and 49 individuals possessed this haplotype. Haplotype 2 was mainly found from the central part of Thailand, and 28 individuals possessed this haplotype. Haplotype 3 was only found in Pilok, and 3 individuals were observed. We compared the nucleotide sequences within and between haplotypes. For 12S and 16S rRNA genes, sequence divergences within haplotypes were 0–0.7% and 0–0.4%, respectively, and among haplotypes were 14.8–18.7% and 10.5–14.8%, respectively (Fig. 3).

To elucidate the phylogenetic relationships of these haplotype groups and other *Fejervarya* species, we carried out MP, ML and BI analyses. Figure 4 shows the resultant ML tree (BP values for the ML and MP analyses). In this ML tree, each haplotype group comprises a clear clade. The haplotype 1 and 2 groups are included in the group that was

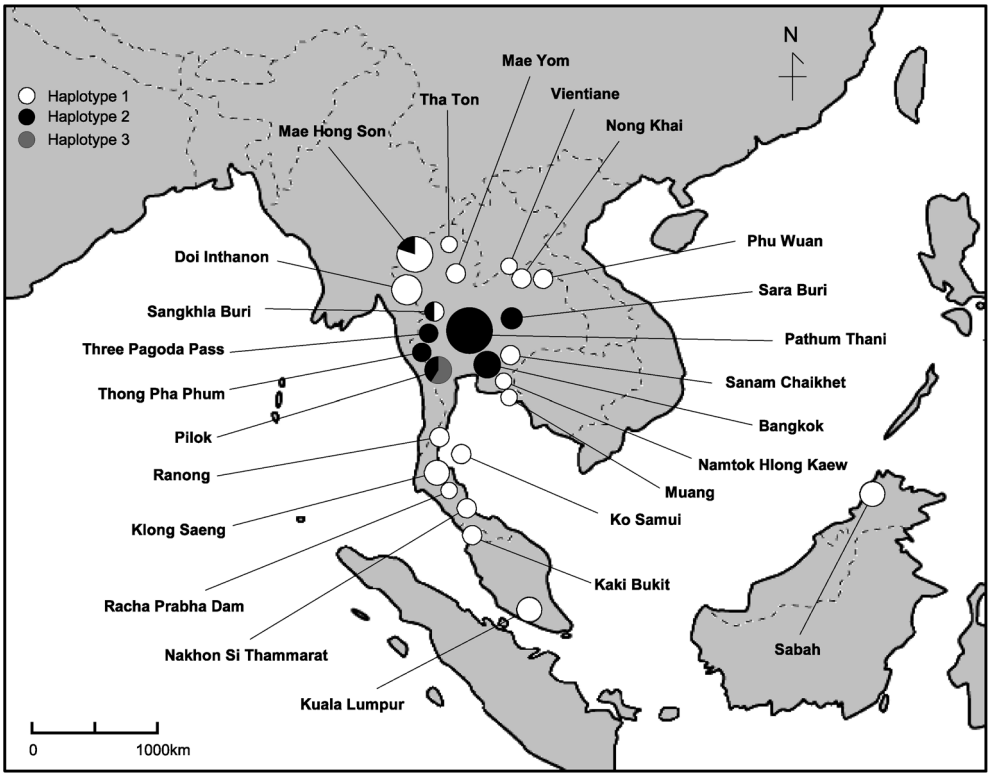

Fig. 2. Map showing the distribution of three haplotypes of the mitochondrial 12S and 16S rRNA genes.

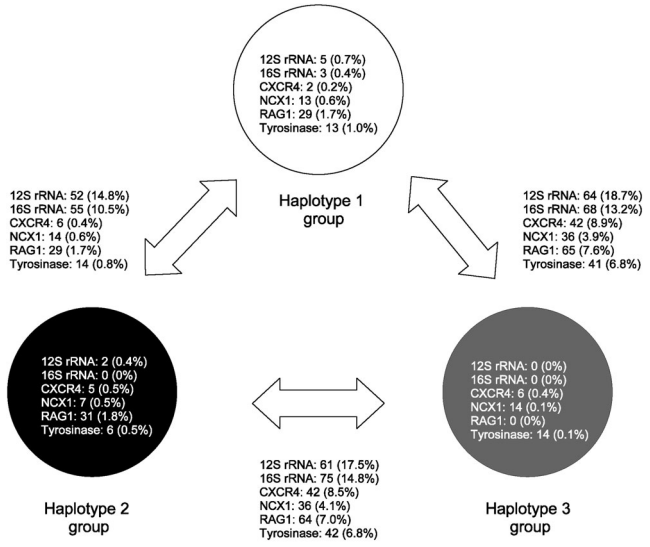

Fig. 3. Three major mitochondrial haplotypes and nucleotide divergences of mitochondrial and nuclear genes. The numbers inside the circles are sequence divergences within haplotypes of mitochondrial and nuclear genes. The numbers outside them are sequence divergences between haplotypes of mitochondrial and nuclear genes.

previously regarded as the Southeast Asian group, whereas the haplotype 3 group is nested in the South Asian group. In the Southeast Asian clade, the haplotype 1 group is monophyletic with *F. limnocharis* (BPs=100/100 and BPP=100). The 12S and 16S rRNA gene sequences of haplotype 1 were almost identical to those of the *F. limnocharis* specimens: there were only one or two changes in the 12S and 16S rRNA genes. The haplotype 2 group and *F. orissaensis* formed a monophyletic group (BPs=91/96 and BPP=97), and the genetic divergence between haplotype 2 and *F. orissaensis* was very low (maximum number of substitutions=6 and average sequence divergence=1.7% for both the 12S and 16S rRNA genes). Haplotype 3 was clearly included in the South Asian group (BPs=99/95 and BPP=100), but there was no corresponding *Fejervarya* species for which mt rRNA sequences have so far been reported. The phylogenetic placements of the three haplotype groups and other intra-relationships of Southeast and South Asian *Fejervarya* species were supported with sufficient statistical significance (see Fig. 4). However, the placements of the genera *Hoplobatrachus* and *Sphaerotheca* were different in each analysis. For example, in the ML tree, *S. dobsoni* diverged at the root of the tree, and *H. tigerinus* and Southeast Asian *Fejervarya* species comprise a monophyletic group (BPs=82/– and BPP=100) (Fig. 4). In the BI tree, *S. dobsoni*, South Asian *Fejervarya*, and Southeast Asian *Fejervarya* show a politomy at the root of the tree, but *H. tigerinus* and Southeast Asian *Fejervarya* species form a monophyletic group as in the ML tree. On the other hand, the MP tree resulted in monophyly of the genus *Fejervarya*, with *S. dobsoni* and *H. tigerinus* branching off at the root of the *Fejervarya* clade. We tested the six alternative phylogenetic hypotheses for the phylogenetic relationships among the South and Southeast Asian *Fejervarya* groups and the

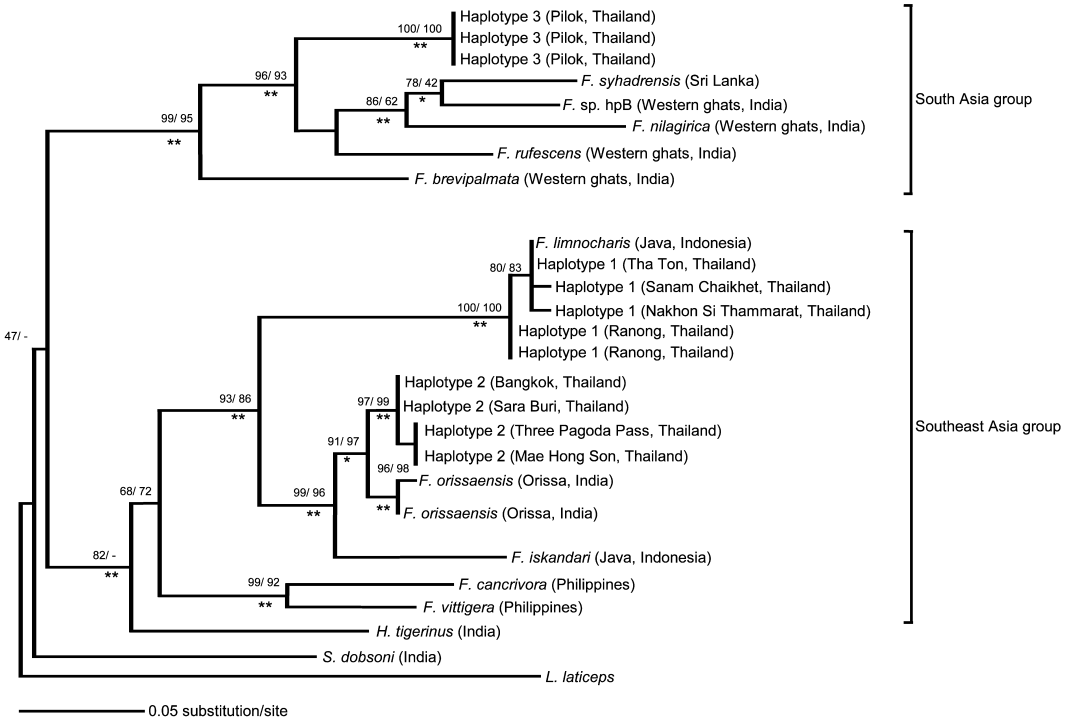

Fig. 4. Maximum-likelihood tree based on 638 bp of the mitochondrial 16S and 12S rRNA genes from 28 frogs. The tree was reconstructed by a heuristic search with PAUP* using the GTR+I+G substitution model suggested by Modeltest. Bootstrap values for ML/MP are shown above the nodes. Asterisks below the branches indicate Bayesian posterior probability (BPP); *>95% and **>99%. Country and locality are shown in parenthesis.

Table 4. Comparison, by KH and SH tests, of log-likelihood scores among alternative tree topologies based on two mitochondrial genes and four nuclear genes.

Tree topology	Method	-ln L	-ln L difference	*P*-value	
				KH	SH
Tree topology based on 2 mitochondrial genes					
(*S. dobsoni*,(South Asia,(Southeast Asia,*H. tigerinus*)))	ML	3554.43869	best tree	–	–
(*H. tigerinus*,(*S. dobsoni*,(Southeast Asia, South Asia)))	MP	3562.09144	7.65275	0.2439	0.1315
(*H. tigerinus*,(Southeast Asia,(South Asia, *S. dobsoni*)))	–	3560.43506	5.99636	0.2458	0.1430
(*S. dobsoni*,(Southeast Asia,(South Asia, *H. tigerinus*)))	–	3562.13806	7.69937	0.1843	0.1093
(*H. tigerinus*,(South Asia, (Southeast Asia, *S. dobsoni*)))	–	3562.27838	7.83969	0.2175	0.1219
((*H. tigerinus*, *S. dobsoni*), (Southeast Asia, South Asia))	–	3562.30885	7.87016	0.2439	0.1315
Tree topology based on 4 nuclear genes					
(*H. tigerinus*,(South Asia,(Southeast Asia,*S. dobsoni*)))	ML	7261.90108	best tree	–	–
(*H. tigerinus*,(Southeast Asia,(South Asia,*S. dobsoni*)))	MP, BI	7262.02397	0.12288	0.9270	0.7311
(*H. tigerinus*,(*S. dobson*,(Southeast Asia,South Asia)))	–	7262.33031	0.42923	0.6781	0.8790
(*S. dobson*,(South Asia ,(*H.tigerinus*,Southeast Asia)))	–	7276.64124	14.74016	0.0264*	0.2981
(*S. dobson*,(Southeast Asia,(*H.tigerinus*, South Asia)))	–	7276.64124	14.74016	0.0264*	0.2981
((*H. tigerinus, S. dobson*),(Southeast Asia,South Asia))	–	7262.33031	14.41332	0.0346*	0.3029

*The values were not significant (< 0.05) among any of the topologies compared.

other related genera by KH and SH tests (Table 4). The KH and SH tests showed no statistically significant differences in log-likelihood values among the six hypothetical topologies, indicating that mt rRNA sequence data could not clarify the relationships between *Fejervarya* and closely related genera.

Nuclear genes

Nucleotide sequences were determined for portions of the CXCR4, NCX1, RAG-1, and tyrosinase genes from the 18 individuals that were used in phylogenetic analyses based on mt genes (Table 3). The sequence divergences of four nuclear genes were 0–1.8% within each haplotype group of the mt rRNA genes (Fig. 3). When we compared sequence divergences of the nuclear genes between haplotype groups and within each group, sequence divergences between haplotype 1 and 3 groups (3.9–8.9%) and between haplotype 2 and 3 groups (4.1–8.5%) showed larger differences than those within group 1 to3 (0.2–1.7%, 0.5–1.8%, and 0–0.4%, respectively). In contrast, the divergences of nuclear genes between haplotypes 1 and 2 (0.4–1.7%) were similar to those between the haplotypes.

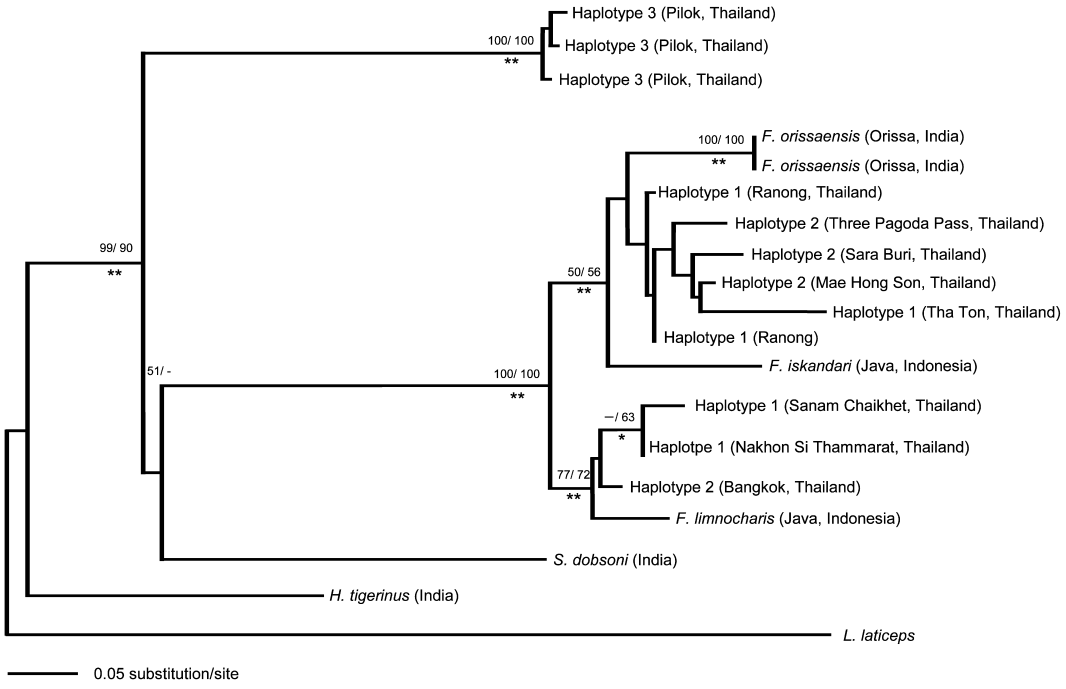

Fig. 5. Maximum-likelihood tree based on 2650 bp sequenced from four nuclear genes from 18 frogs. The tree was reconstructed by a heuristic search with PAUP* using the GTR+I+G substitution model suggested by Modeltest. Bootstrap values for ML/MP are shown above the nodes. Asterisks below branches are Bayesian posterior probability (BPP); *>95% and **>99%. Country and locality are shown in parenthesis.

The resultant MP, ML, and BI trees (Fig. 5) showed that the haplotype 1 and 2 groups were included in the Southeast Asian group, as was the case in mt gene trees, and that the haplotype 3 group (member of the South Asian group) was divergent from the Southeast Asian group. However, in the nuclear gene trees, the haplotype 1 and 2 groups did not form a clade, and individuals of these haplotypes were scattered in the Southeast Asian clade; e.g., the haplotype 1 group from Ranong made a clade with the haplotype 2 group, *F. orissaensis*, and *F. iskandari*, and the haplotype 2 group from Bangkok formed a clade with *F. limnocharis* and haplotype 1 groups from Sanam Chaikhet and Nakhon Si Thammarat.

In the nuclear gene trees, the genus *Hoplobatrachus* constantly diverged at the root of the trees (Fig. 5). However, the placement of the genus *Sphaerotheca* was different among MP, ML, and BI trees; the ML tree showed the monophyly of *S. dobsoni* and Southeast Asian *Fejervarya* species (BP=51) (Fig. 5), whereas the MP and BI trees weakly support an *S. dobsoni* and haplotype 3 clade (BPP=67). KH and SH tests for the six alternative hypotheses of intergeneric relationships indicated that there were no statistically significant differences in log-likelihood values among these trees (Table 4). Therefore, the nuclear gene data also failed to clarify the intergeneric relationships.

DISCUSSION

Recent molecular phylogenetic studies indicate that the genus *Fejervarya* is divided into two main groups: the *F. limnocharis* group distributed in Southeast and East Asia and the *F. syhadrensis* group distributed in India and South Asia (Kurabayashi et al., 2005; Frost et al., 2006; Sumida et al., 2007). Our mt gene data shows that the haplotype 1 and 2 groups were included in the Southeast Asian group and that haplotype 3 was nested in the South Asian group (Fig. 4). Based on mt gene data, in the Southeast Asian group, the haplotype 1 group made a clade with *F. limnocharis* collected from the type locality of this species. The maximum sequence divergences between haplotype 1 and *F. limnocharis* were 0.9% and 0.6% for 12S and 16S rRNA genes, respectively. This small sequence divergence of mt genes and the resultant phylogenetic relationship clearly indicate that the haplotype 1 group, which is widely distributed in Thailand, corresponds to the "real" *F. limnocharis*.

The haplotype 2 group, which is widely distributed in the central part of Thailand, formed a clade with *F. orissaensis* distributed in Orissa in India (Fig. 4). The very small nucleotide divergence of mt genes (1.7% for both the 12S and 16S) between the haplotype 2 group and *F. orissaensis* and their monophyletic relationship suggest that the haplotype 2 group is the same as *F. orissaensis*, though we should await further morphological analyses of this haplotype group. This result is also congruent with that of Sumida et al. (2007), who demonstrated that "*F. limnocharis*" from Bangkok (=haplotype 2 group) has a very close relation to *F. orissaensis*. However, there was no difference in external morphology between Ranong (haplotype 1 group) and Bangkok (haplotype 2 group) samples (Sumida et al., 2007).

Based on the mt genes, the haplotype 3 group was phylogenetically nested in South Asia rather than Southeast Asia. Furthermore, the individuals of haplotype 3 group were smaller than those of typical Southeast Asian *Fejervarya* groups (including the haplotype 1 and haplotype 2 groups), and the haplotype 3 group could be distinguished morphologically from the haplotype 1 and haplotype 2 groups (Djong et al., 2007). At present, 15 *Fejervarya* species possibly belonging to the South Asian group (=*F. syhadrensis* group) are known (Frost, 2006), and 16S sequences are available from eight of the 15 species. The 16S sequence of the haplotype 3 group does not match any reported 16S sequences of the South Asian species. Therefore, to check whether the haplotype 3 group corresponds to a described or an undescribed species, intensive sampling of the South Asian taxa will be needed.

Although the phylogenetic analyses based on mt genes showed that each haplotype group comprised a distinct clade (Fig. 4), the nuclear gene data did not support the monophyly of each haplotype, but rather showed random placements of individuals of haplotypes 1 and 2 in the Southeast Asian clade (Fig. 5). The possible reasons for the different results between the mt and nuclear data are considered to be as follows: (I) the haplotype 1 and 2 groups (and *F. limnocharis*, *F. iskandari*, and *F. orissaensis*) are not different species, and hybridize naturally and frequently. In this case, there are two different mitochondrial types in the same species. (II) The rate of nucleotide substitutions of the nuclear genes was very low, and polymorphic sites that emerged in the ancestors of the Southeast Asian groups were maintained in their offspring even after speciation. In addition, not enough time has passed to fix the nucleotide sites unique to each species or species group. This case is well known as an effect of ancestral polymorphism. With regard to (I), Djong et al. (2007) carried out hybridization experiments between *F. limnocharis* (=haplotype 1 group) and *F. iskandari* (with a close relationship with the haplotype 2 group; see Fig. 4), and reported incomplete postmating isolation between them. Moreover, it is known that *F. limnocharis* and *F. iskandari* occur sympatrically in some localities in Indonesia, but never hybridize (Toda et al., 1998). Sumida et al. (2007) also conducted hybridization experiments between the Ranong (=haplotype 1 group) and Bangkok (=haplotype 2 group) populations, and found insufficient growth in the hybrid larvae. For these reasons, it is unlikely that the haplotype 1 and 2 groups are a natural hybrid species or a species hybridized with high frequency and, if hybridization is possible, it is extremely difficult for the hybrids to grow. Consequently, the possibility of hypothesis (I) is low.

Next, to examine the possibility of hypothesis (II), polymorphic loci in the first and second codon positions in the nuclear genes were closely examined, because multiple nucleotide substitutions seem to be rare at the first and second codon sites. As a result, many sites showing the possibility of ancestral polymorphisms were found. While there were 121 variable sites in all 1758 first and second codon sites, 43 sites were characteristic of the Southeast Asian group; the remaining 78 variable sites were observed only in the haplotype 3 group and non-*Fejervarya* taxa). Twenty-nine sites in 43 were autapomorphic substitutions that were observed in only one individual. For the remaining 14 sites, there were no synapomorphic nucleotides between the haplotype 1 group and *F. limnocharis* or between the haplotype

2 group and *F. orissaensis*. In contrast, at almost all these sites, the same substitutions occurred across the haplotype 1 and 2 groups. For example, at site 273 of the RAG-1 gene, guanine (G) seemed to be symplesiomorphic, and a derived adenine (A) nucleotide was found in both the haplotype 1 and haplotype 2 groups. At site 139 of the tyrosinase gene, there were adenine (A) and cytosine (C) nucleotides, and their heterozygous sites were found in some individuals of both the haplotype 1 and haplotype 2 groups. For these reasons, it is highly possible that many ancestral polymorphic sites remain in nuclear genes of the Southeast Asian *Fejervarya* species. Thus, the nuclear gene data failed to elucidate the phylogenetic relationships of Southeast Asian *Fejervarya* taxa. As mentioned in the Introduction, although a possible introgression of mt DNA due to hybridization has been suggested, this was not supported by the present study. However, the sites of the nuclear genes we used were different from those used in the allozyme analyses. Accordingly, the reason for the incongruous results between the analyses using mt genes and allozymes remains unknown. We should therefore collect more samples from Ranong and Bangkok, and carry out allozyme analyses in detail. Another efficient approach would be to examine the distribution patterns of polymorphisms in nuclear genes encoding enzymes used in allozyme analysis.

According to Frost et al. (2006), the genus *Fejervarya* should, for the time being, be recorded as "*Fejervarya*" with parentheses for expedience, because their analysis suggested paraphyly of the genus *Fejervarya* based on long sequences of mt genes (2400 bp) and nuclear genes (2300 bp). Specifically, their phylogenetic tree showed that *Hoplobatrachus*, *Euphlyctis,* and the South Asian *Fejervarya* species formed a clade, to which *Sphaerotheca* formed a sister group, with the Southeast Asian *Fejervarya* species as the sister group to the above clade. In this study, the ML tree from mt genes showed that *H. tigerinus* and Southeast Asian *Fejervarya* species formed a clade (BPs=82/–), and the other South Asian *Fejervarya* species were the sister group to this clade (=*Hoplobatrachus*+ Southeast Asian group) (Fig. 4). However, the BP values supporting this relationship were low (BPs=47/–). BI analysis also showed the monophyly of *H. tigerinus* and Southeast Asian *Fejervarya* species (BPP=100), but the relationship among this clade, South Asian *Fejervarya*, and *S. dobsoni* was not elucidated (forming a polytomy). The KH and SH tests also showed no statistically significant differences among any of the topologies compared (Table 4). Therefore, the data from the mt genes used here did not clarify the phylogenetic relationships between the genus *Fejervarya* and its related genera. On the other hand, based on the nuclear data, MP, ML and BI methods supported the nested placement of *Sphaerotheca* in "*Fejervarya*." Although the BP value and BPP of the *Fejervarya* and *Sphaerotheca* clade were low (BPs=51/– and BPP=67) and the nuclear data could not identify the exact placement of the genus *Sphaerotheca*, the KH and SH tests rejected the monophyly of "*Fejervarya*" (see Table 4). Thus, the results from nuclear gene data seems to suggest the paraphyly of "*Fejervarya*" with respect to the genus *Sphaerotheca*.

As in previous studies (Kurabayashi et al., 2005; Frost et al., 2006; Sumida et al., 2007), in our results the genus *Fejervarya* was divided into two major clades of the Southeast and South Asian groups (Fig. 4 and 5). A possible geographical barrier between these areas is the mountain arc that stretches from the Arakan Mountain Range to the Patkai Mountains. However, our phylogenetic analyses concurrently showed that *F. orissaensis*, which is known only from Orissa in India, was included in the Southeast Asian group, and that the haplotype 3 group from Pilok in Thailand was included in the South Asian group (Fig. 4). Although *F. orissaensis* has been reported only from India, the haplotype 2 group that is possibly the same species as *F. orissaensis* is widely distributed in Thailand (Fig. 2). Furthermore, the clade of *F. orissaensis* and the haplotype 2 group form a sister-group relationship in the Southeast Asian group (Fig. 4). The haplotype 3 group was observed only in the western part of Thailand, which is geographically close to India, and our results showed that the haplotype 3 group is a member of the South Asian group (Fig. 4). These results and distribution patterns suggest that: (1) the origin of *F. orissaensis* was somewhere in Southeast Asia, and *F. orissaensis* (or its ancestor) spread to South Asia, and (2) the origin of the haplotype 3 group lies in South Asia, especially in India, and spread to Southeast Asia. Therefore, the Arakan and Patkai Mountains were perhaps not the cause of the division between the South and Southeast *Fejervarya* groups. To investigate what caused the phylogenetic divergence of these two groups, detailed phylogenetic analyses with more samples and better estimates of divergence time are required. Geographic events that occurred around the estimated time of divergence of the Southeast and South Asian groups should then be examined.

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Appendix 1. Specimens used in this study. For abbreviations of museum and institution, refer to the text.

Thailand: Tha Ton (KUHE 19879, 19880, 19913), Mae Hong Son (KUHE 19821, 19839–19843, 19846, 19863), Doi Inthanon (KUHE 19003, 19024, 19059, 19099, 19138, 19139), Mae Yom (KUHE 21997, 22001), Three Pagoda Pass (KUHE 19504, 19505), Pilok (KUHE 35196–3518, IABHU 32652, 32714), Ko Samui (KUHE 19608, 19609), Klong saeng (KUHE 19641, 19642, 19670, 19671), Racha Prabha Dam (KUHE 19658), Nakhon Si Thammarat (KUHE 19338, 19385), Sanam Chaikhet (KUHE 19804, 19805), Nong Khai (KUHE 22140, 22141), Phu Wuan (KUHE 22270, 22279), Muang (KUHE 34065), Namtok Hlong Kaew (KUHE 34104), Sangkhal Buri (KUHE 19482, 19469), Bangkok (IABHU 32473, 32474, 32490–32492), Ranong (IABHU 32488, 32489), Thong Pha Phum (IABHU 32553), Sara Buri (IABHU 32648, 32709), Pathum Thani (IABHU 32650, 32685–32692).

Malaysia: Kaki Bukit (KUHE 35464, 35465), Sabah (IABHU 32710, 43053), Kuala Lumpur (IABHU 32684, 18131, 18150, 32649).

Laos: Vientiane (KUHE 34310).